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Genetic variation of the ghrelin activator gene ghrelin O-acyltransferase (*GOAT*) is associated with anorexia nervosa

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ABSTRACT

The gastrointestinal peptide hormone ghrelin promotes food intake and increases body weight and adiposity through activation of the growth hormone secretagogue receptor (GHSR1a). To promote its biological action ghrelin is acylated at its serine 3 residue by the recently discovered ghrelin O-acyltransferase (GOAT, a.k.a. membrane-bound O-acyltransferase 4, MBOAT4). Plasma levels of total and acyl-ghrelin are negatively correlated with body-mass-index (BMI); as lower the BMI as higher plasma levels of total and acylated ghrelin and vice versa. Accordingly, plasma levels of total and acyl-ghrelin are elevated in patients with anorexia nervosa (AN) and decline upon weight regain. The importance of the endogenous Goat/ghrelin system in the neuroendocrine adaptation to fasting was recently highlighted by the observation that acyl-ghrelin mediated elevation of growth hormone (GH) release prevents starvation induced hypoglycemia in *Goat*$^{-/-}$ mice. The aim of this study was to test if genetic variation of *GOAT* is implicated in the etiology of AN. We therefore assessed association of 6 tagging single nucleotide polymorphisms (tagSNPs), which were predicted to cover 96% the common genetic variability of *GOAT* plus 50 kb of the 5′ and 3′ flanking region, in 543 German patients with AN and 612 German normal and underweight healthy controls. Based on a recessive mode of inheritance we observed some evidence for association of the G/G genotype at SNP rs10096097 with AN (nominal two-sided $p = 0.031$). Based on our results we conclude that genetic variation in *GOAT* might be implicated in the etiology of AN.
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1. Introduction

Anorexia nervosa (AN) is an eating disorder of unknown etiology, affecting approximately 1–2% of adolescents, mostly young women between the ages of 14 and 18 years (Vyver et al.,

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2008; Hoek and van Hoeken, 2003). Highly efficacious or even curative pharmacological treatment options remain to be discovered. Even though substantial evidence indicates that approximately 50–80% of the variance of AN can be explained by genetic factors (Klump et al., 2001), little is known about the molecular mechanisms of this disorder. Several studies have suggested that genes implicated in the regulation of food intake and energy metabolism might be implicated in the etiology of AN (Hebebrand and Remschmidt, 1995; Hinney et al., 2004; Pinheiro et al., 2006; Sulek et al., 2007; Scherag et al., 2010).

The gastrointestinal peptide hormone ghrelin promotes body weight gain and adiposity by leading to increased food intake and decreased fat utilization in both rodents (Tschöp et al., 2000; Asakawa et al., 2001) and humans (Wren et al., 2001; Hotta et al., 2009; Druce et al., 2006). Numerous studies are currently revealing ghrelin's mechanisms of action, which are believed to rely on specific hypothalamic as well as extra-hypothalamic neurocircuitries. Until recently however, relatively little was known about the mechanisms of ghrelin activation, as the hormone's ability to bind and activate its known receptor (GHSR1a) depends on post-translational acylation of the ghrelin peptide at the serine 3 residue (Kojima et al., 1999). Recently, the membrane-bound O-acyltransferase 4 (MBOAT4), which was later renamed to ghrelin O-acyltransferase (GOAT), was identified as the endogenous key enzyme responsible for ghrelin octanoylation (Yang et al., 2008; Gutierrez et al., 2008). The observation that acylated ghrelin is completely absent in mice lacking *Goat* (*Goat*$^{-/-}$) indicates that Goat is the only enzyme capable of acylating ghrelin in-vivo (Kirchner et al., 2009).

In rodents, plasma levels of ghrelin are increased in response to fasting and decrease upon re-feeding (Tschöp et al., 2000; Asakawa et al., 2001; Kirchner et al., 2009). Accordingly, plasma levels of total and acyl-ghrelin are elevated in patients with AN (Otto et al., 2001; Nakahara et al., 2008; Germain et al., 2007, 2009; Prince et al., 2009; Monteleone et al., 2008; Tolle et al., 2003) and decline to normal values upon weight restoration (Otto et al., 2001, 2005; Troisi et al., 2005). Interestingly, several small studies reported higher plasma levels of acyl-ghrelin in patients with AN when compared to BMI-matched lean controls (Germain et al., 2007, 2009; Tolle et al., 2003).

Ghrelin promotes food intake through stimulation of hypothalamic orexigenic neuropeptides, such as neuropeptide Y (Npy) and agouti-related protein (Agrp) (Kamegai et al., 2000, 2001). Despite its orexigenic effects ghrelin promotes the expression and release of growth hormone (GH) from the anterior pituitary gland and thus indirectly triggers the release of hepatic insulin-like growth factor-1 (IGF-1) (Kojima et al., 1999; Arvat et al., 2001). Both, GH and IGF-1 are anabolic hormones that increase lean body mass by stimulation of skeletal muscle growth and inhibition of skeletal muscle protein breakdown (Fuller et al., 1992; Moller et al., 2009). Resistance to GH is typically accompanied by high plasma levels of GH and low plasma levels of IGF-1 (Hanaire-Broutin et al., 1996; Monzavi and Cohen, 2002). Impairment of the GH/IGF-1 axis is frequently described in patients with AN; plasma levels of GH are typically elevated while IGF-1 levels are decreased (Counts et al., 1992; Scacchi et al., 1997; Misra et al., 2003). The importance of the Goat/ghrelin system in the modulation of the GH/IGF-1 axis was recently demonstrated by Zhao and colleagues. They showed in *Goat*$^{-/-}$ mice that ghrelin mediated elevation of GH prevents death by preserving blood glucose levels under conditions of severe caloric restriction (Zhao et al., 2010). Together, the observation that plasma levels of total and acyl-ghrelin are increased and that the GH/IGF-1 axis is impaired in patients with AN indicates that genetic variation of *GOAT* might be implicated in the etiology of AN. The aim of the present case-control association study was therefore to assess the potential implication of genetic variation in *GOAT* with AN.

Table 1
Genotype and allele frequencies of the 6 analyzed tagSNPs of the *GOAT* gene in 543 German patients with anorexia nervosa (AN) and 612 German normal and underweight healthy controls.

			AN Cases				Controls				
SNP	Alleles[a]	Location	N	Genotypes N (%)	Alleles %	HWE p-value	N	Genotypes N (%)		Alleles %	HWE p-value	p-values[b]
rs10096097	−24.981 A/G	5′ UTR	541	AA 312 (57.67)	A: 74.31	0.003	610	AA	332 (54.43)	A: 74.34	0.339	a: 0.984
				AG 180 (33.27)				AG	243 (39.84)			d: 0.268
				GG 49 (09.06)	G: 25.69			GG	35 (05.74)	G: 25.66		r: 0.031
rs4733400	−10.397 A/G	5′ UTR	541	AA 466 (86.14)	A: 92.61	0.201	610	AA	515 (84.43)	A: 91.89	1	a: 0.524
				AG 70 (12.94)				AG	91 (14.92)			d: 0.414
				GG 5 (00.92)	G: 07.39			GG	4 (00.66)	G: 08.11		r: 0.606
rs13272159	12.719 A/G	Exon 3	541	AA 261 (48.24)	A: 67.84	0.016	610	AA	278 (45.57)	A: 67.13	0.582	a: 0.726
				AG 212 (39.19)				AG	263 (43.11)			d: 0.365
				GG 68 (12.57)	G: 32.16			GG	69 (11.31)	G: 32.87		r: 0.511
rs1355412	16.435 A/G	3′ UTR	543	AA 418 (76.98)	A: 87.48	0.334	612	AA	461 (75.33)	A: 87.17	0.203	a: 0826
				AG 114 (20.99)				AG	145 (23.69)			d: 0.511
				GG 11 (02.03)	G: 12.52			GG	6 (00.98)	G: 12.83		r: 0.141
rs16876504	26.800 A/G	3′ UTR	541	AA 457 (84.47)	A: 91.87	0.812	610	AA	517 (84.75)	A: 92.30	0.245	a: 0.700
				AG 80 (14.79)				AG	92 (15.08)			d: 0.895
				GG 4 (00.74)	G: 08.13			GG	1 (00.16)	G: 07.70		r: 0.139
rs3735989	31.214 C/G	3′ UTR	541	CC 281 (51.94)	C: 71.07	0.097	610	CC	295 (48.36)	C: 70.33	0.208	a: 0.696
				CG 207 (38.26)				CG	268 (43.93)			d: 0.225
				GG 53 (09.80)	G: 28.93			GG	47 (07.70)	G: 29.67		r: 0.209

[a] Numbers are given according to genomic entry NM_001100916 and the *GOAT* translation start codon (nt + 1 is the A of ATG). SNP alleles correspond to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).
[b] *p*-value in the Cochrane-Armitage trend test for the (a) log-additive (d) dominant and (r) recessive model for the minor allele.

2. Methods

2.1. Study subjects

The ascertainment strategy was previously described in detail (Hinney et al., 1997, 2006). Written informed consent was given by all participants and in the case of minors, by their parents. The study was approved by the Ethics Committees of the Universities of Marburg and Duisburg-Essen, Aachen, Tübingen, Heidelberg, Erlangen, Freiburg, Würzburg, Bochum and the Charité Berlin, and carried out according to the Declaration of Helsinki.

Study group 1 (AN cases) comprised 543 (1 male) German patients with AN. All patients with AN were recruited based on the DSM-IV criteria for anorexia nervosa (American Psychiatric Association, APA, 1994). Of these, 460 (1 male) individuals had acute AN, whereas 81 (0 males) were recovered individuals from long-term follow-up studies of AN (Herpertz-Dahlmann et al., 1996, 2001; Zipfel et al., 2000). At inclusion, the acute patients had a mean age of 19.79 ± 7.80 years and a mean BMI of 15.68 ± 1.74 kg/m^2. The recovered patients had a mean age of 27.89 ± 9.49 years and a mean BMI of 20.72 ± 2.57 kg/m^2. In total, the 543 patients with AN had a mean age of 21.00 ± 8.57 years and a mean BMI of 16.44 ± 2.61 kg/m^2 (mean BMI percentile based on the German National Nutrition Survey (Hebebrand et al., 1994; Anders et al., 1990) of acute and recovered AN patients was 3.49 ± 7.93 and 38.80 ± 28.78, respectively).

Study group 2 (controls) comprised 612 German normal and underweight healthy controls (229 males) with a mean age of 25.66 ± 5.13 years and a mean BMI of 19.11 ± 1.91 kg/m^2 (mean BMI percentile 15.87 ± 19.55) (Müller et al., 2010). All controls with a current eating disorder like AN or Eating Disorder not Otherwise Specified (EDNOS), as assessed by a short version of the composite international diagnostic interview (CIDI) were excluded from the study. The CIDI questionnaire was kindly provided by Prof. Wittchen.

2.2. Molecular genetic methods

Using the 'Algorithm-Tagger-Pairwise Tagging' approach implemented in HapMap (www.hapmap.org, HapMap Data Release 24/PhaseII Nov08, NCBI36 assembly, r^2 cut-off: 0.8, minor allele frequency (MAF): 0.1), six tagSNPs (rs1355412, rs10096097, rs16876504, rs3735989, rs13272159 and rs4733400) were predicted to cover 96% of the common genetic variability of the *GOAT* coding regio ± 50 kb of the 5′ and 3′ flanking region as derived from the HapMap-CEU population. The analyzed tagSNPs are located in the 5′ flanking region (rs10096097, rs4733400), exon 3 (rs13272159) or the 3′ flanking region (rs1355412, rs16876504, rs3735989) of *GOAT* (Table 1).

Genotyping was performed using restriction-fragment length polymorphism analyses (PCR-RFLP). Only SNP rs13272159 was genotyped using tetra-ARMS-PCR (Ye et al., 2001) as described previously (Müller et al., 2008a). The corresponding primer sequences and restriction enzymes are displayed in Table 2. For validity of the genotypes, alleles were rated independently by at least two experienced individuals. Discrepancies were resolved unambiguously either by reaching consensus or by retyping. Additionally, several random samples of patients being homozygous for the low frequency alleles of SNPs rs10096097 and rs13272159 were resequenced to confirm reliability of genotyping.

2.3. Statistics

The genotype distribution of the analyzed variants was tested for deviations from Hardy–Weinberg equilibrium using an exact two-sided test (Wigginton et al., 2005). As we had no specific a priori hypothesis for the underlying mode of inheritance we used the Cochran–Armitage trend test for the log-additive, recessive and the dominant mode of inheritance (Armitage, 1955) to test for association with AN.

Additionally, odds ratios (OR) and 95% confidence intervals (95% CI) were estimated based on a logistic regression with and without adjustment for sex for a 2-parameter model and three 1-parameter models for which each genotype was compared to the other genotypes. All reported *p*-values are two-sided, nominal, not adjusted for multiple testing and if not stated otherwise based on asymptotical considerations.

Power calculations were performed using the program QUANTO version 1.2.4 under the assumption of a MAF of 0.3 as observed in the HapMap-CEU sample for rs10096097. 543 cases and 612 controls were estimated to yield a power >0.80 to detect an OR of 1.28, 1.68 and 1.39 for a log-additive, recessive and dominant model with the minor allele as risk allele, respectively.

3. Results

Analysis of 6 tagSNPs in 1155 individuals (543 German patients with AN and 612 German normal and underweight healthy controls) revealed some evidence for an association of the G/G genotype at SNP rs10096097 with AN (recessive model; nominal two-sided $p = 0.031$, Table 1). The corresponding genotypic odds ratio (OR) and 95% confidence interval (95% CI) for the G/G

Table 2
Primer sequences and restriction enzymes.

SNP	Primer (5' - 3')	Product size	Enzyme[b]	RFLP-PCR Fragment size
rs10096097	F: 5′-AGC CAG TAA GGC AGT TCC AA-3′ R: 5′-TCA CCA CGG AAA ACA TTG AA-3′	153bp	HhaI	G: 114bp + 39bp A: 153bp
rs4733400	F: 5′-GGT GGA GAG TCA GCT TTG CT-3′ R: 5′-ACA CAG CAT GAT CCC GTC TC-3′	235bp	DraI	A: 133bp + 102bp G: 235bp
rs1355412	F: 5′-GAG CAA TGG GCA GAG TCT TG-3′ R: 5′-GAT GGC CAG GGG TAA TTT CT-3′	396bp	Hpy188I	G: 145bp, 128bp, 96bp, 27bp A: 241bp, 128bp, 27bp
rs13272159[a]	F-in: 5′-AGG AAA CAT TTT AGA AAG TGC CAG ATT AC-3′ R-in: 5′-AAA GTT CAA ATG TAT GCA TTA TCG ATT CA-3′ F-out:5′-AGT GTC TTT CCC ATG GTG TAC TGT ATT C-3′ R-out:5′-GTA AAC CTT AAG GGA AGA AAC CAC ATC T-3′			
rs16876504	F: 5′-AGA GCA TCA GCC AGG ACATT-3′ R: 5′-AAC CAC CTA ATA CAG TCA CAG TTG A-3′	150bp	FauI	G: 104bp + 46bp A:150bp
rs3735989	F: 5′-ACG CTT GCT AGC AGC ATT TT-3′ R: 5′-GGA GGC AGA GGT TGC AGT AA-3′	232bp	BtscI	C: 187bp + 45bp G: 232bp

[a] Product size of outer primer is 302bp, product size for C and T-allele is 203bp and 157bp, respectively.
[b] All enzymes have been purchased from NEB, Germany.

Table 3
Odds ratios and 95% confidence intervals for SNP rs10096097 of GOAT based on a logistic regression analysis with and without adjustment for sex.

Genotype/Model	OR	95% CI	*p*-value	Adj. OR[a]	Adj. 95% CI[b]	Adj. *p*-value[c]
A/A	1			1		
A/G	0.788	0.616–1.009	0.059	0.824	0.623–1.090	0.174
G/G	1.490	0.940–2.361	0.090	2.014	1.121–3.319	0.019
Recessive Model: G/G vs A/G + A/A	1.636	1.043–2.566	0.032	2.172	1.221–3.862	0.008

[a] Sex adjusted odds ratio (OR).
[b] Sex adjusted 95% confidence interval (CI).
[c] Sex adjusted *p*-value.

genotype compared to G/A and A/A based on a logistic regression is OR 1.613, 95% CI 1.037–2.508, $p = 0.032$, Table 3. This effect was stable after stratification for sex (Table 3). Noteworthy, the association of the G/G genotype at SNP rs10096097 with AN was stable after exclusion of male subjects (data not shown).

Notably, a trend towards deviation from Hardy–Weinberg equilibrium was observed for genotype frequencies of SNPs rs10096097 and rs13272159 in AN cases ($p = 0.002$ and $p = 0.016$, respectively) but not in controls ($p = 0.43$ and $p = 0.48$, Table 1). This finding, however, is not surprising and expected in case of a true genetic association and indeed the number of homozygotes for the rs10096097 risk G-allele was increased in AN patients compared to controls (frequency of risk G/G genotype in AN cases and controls is 9.24% and 5.94%, respectively; Table 1). However, to ensure validity of genotyping all homozygous G-allele carrier at rs10096097 and rs13272159 were re-genotyped. Additionally, several random samples were resequenced. The genotypes were in 100% accordance.

4. Discussion

Ghrelin is the only circulating molecule to significantly promote food intake and body weight, which it achieves through activation of the ghrelin receptor (GHSR1a). Recently, the ghrelin O-acyltransferase (GOAT) was identified as the endogenous key enzyme responsible for ghrelin activation (Yang et al., 2008; Gutierrez et al., 2008).

The human *GOAT* gene is located on chromosome 8p12 and comprises three exons spanning 13.02kb. The human transcript contains 1.308kb which are translated into a protein of 435 amino acids from which Asp307 and His338 are essential for the enzymes ability to octanoylate ghrelin (Yang et al., 2008; Gutierrez et al., 2008). The GOAT protein is highly conserved between mammals and the expression is largely restricted to ghrelin expressing tissues, such as the stomach and the intestine (Yang et al., 2008; Kirchner et al., 2009). Due to its key role in mediating ghrelin acylation, GOAT may offer potential in the treatment of eating/wasting disorders and cachexia.

Several studies have shown an inverse relation between the body-mass-index (BMI) and circulating levels of acylated ghrelin (Tolle et al., 2003; Nakai et al., 2004; Harada et al., 2008). Accordingly, compared to normal weight healthy controls, plasma levels of acyl-ghrelin are elevated in patients with AN, even during all phases of an oral glucose tolerance test (Nakai et al., 2004; Harada et al., 2008).

Here we describe to our knowledge, the first analysis of the potential relevance of genetic variation in the human *GOAT* gene and anorexia nervosa. Analysis of 6 tagging SNPs in 543 German patients with AN and 612 German normal and underweight healthy controls revealed some evidence for association of the G/G genotype at SNP rs10096097 with AN (recessive model, nominal $p = 0.031$). The corresponding logistic regression analysis showed that homozygous risk G-allele carriers have an increased risk for AN. However, the potential implication of SNP rs10096097, which is located 24.981bp upstream the translation start codon (ATG) of *GOAT*, for AN remains unknown. Gain-of-function mutations in *GOAT* might, at least in part, account for the observed higher plasma levels of acyl-ghrelin and growth hormone in patients with AN. Persistent hyperghrelinemia might entail ghrelin and growth hormone resistance, comparable to insulin resistance in persistently hyperinsulinemic individuals. The observation that patients with AN show higher plasma levels of acyl-ghrelin compared to BMI-matched healthy controls (Germain et al., 2007, 2009; Troisi et al., 2005) supports this hypothesis. On the other hand loss-of-function mutations in *GOAT* might lead to impaired eating behavior and thus an increased risk for AN.

Several association studies for AN have previously focused on genes implicated in the regulation of energy balance, such as on the genes coding for ghrelin (Monteleone et al., 2006; Cellini et al., 2006; Dardennes et al., 2007; Gueorguiev et al., 2009), the ghrelin receptor (Gueorguiev et al., 2009; Miyasaka et al., 2006), leptin (Hinney et al., 1998), the leptin receptor (Quinton et al., 2004), the brain derived neurotrophic factor (*BDNF*) (Ribasés et al., 2003, 2004, 2005; Friedel et al., 2005), the tumor necrosis factor-alpha (Ando et al., 2001) or genes of the endocannabinoid system (Müller et al., 2008b). Most of these studies have yielded negative results.

Eating disorders are typically characterized by diagnostic instability, and migration between different subtypes, and even between different eating disorders (Milos et al., 2005; Tozzi et al., 2005). Consistency of diagnostic criteria over several months is therefore a requirement to clearly distinguish between different AN subtypes. Accordingly, one of the limitations of our study is that detailed amnesic information, especially regarding previous eating disorders as well as follow-up data, were not available for all samples. However, whereas several studies consistently show that 25–30% of patients with eating disorders migrate from AN to BN, migration from BN to AN is less frequently described (Tozzi et al., 2005; Gorwood et al., 2003). Accordingly, it is unlikely that the observed association of the rs10096097 G/G genotype with AN can be explained by AN patients with a history of BN. In summary we here report some evidence for an association of the G/G genotype at SNP rs10096097 5′ to the *GOAT* gene with AN. Additional studies in larger samples are warranted to confirm our data.

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